A new diagnostic platform for prediction of drug response based on a tumor's microRNA profile

Rolf Søkilde*, Poul Erik Høiby*, David L Smith**, William Ricketts**, Søren Møller*, Thomas Litman*
*Exiqon A/S, Skelstedet 16, DK-2950 Vedbæk, Denmark. **Oncotech, 15501 Red Hill Avenue, Tustin CA 92780

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Introduction

Increasing evidence suggests that microRNAs play a key role in the initiation and progression of cancer, and therefore, may comprise a novel class of molecular biomarkers with prognostic and predictive potential.

Drug resistance is a major impediment to the successful chemotherapeutic treatment of cancer. Current tests assay the *ex vivo* growth of a tumor in the presence of chemotherapeutic drugs but new molecular tests are needed. We have analyzed 70 colorectal cancer specimens that had been previously tested in the Extreme Drug Resistance assay, a clinically validated drug resistance test (Figure 1). Each sample's microRNA expression pattern was assayed on an LNA™ (Locked Nucleic Acid) enhanced microarray platform, which allows for very sensitive and specific detection of small RNA targets like microRNAs.

This study, for the first time demonstrates that the microRNA expression pattern of a tumor biopsy reflects the drug resistance status of the cancer. Our analysis enabled identification of tumors that were resistant to 5-FU/Leucovorin, Oxaliplatin, or Irinotecan, based solely on the tumor cell's microRNA profiles. Thus, we propose that a molecular Drug Resistance (mDR) assay, based on a tumor's microRNA profile can potentially provide a basis for prediction of drug response. Two advantages of such an mDR assay are that it requires very little amount of input RNA (down to 100 ng), and its fast (one day) response time.

Figure 1

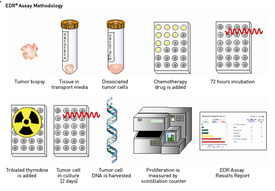


Figure 1. Our R&D team at Oncotech uses the EDR Assay results as a surrogate marker for clinical resistance, thus allowing us to rapidly identify and develop new cancer drug response diagnostics.

Figure 2

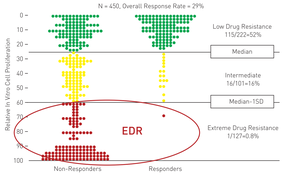


Figure 3

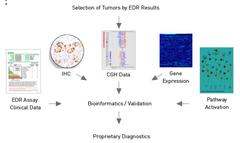


Figure 3. The EDR assay enables highly specific prediction of antineoplastic drug resistance.

Methods

Samples

70 formalin fixed, paraffin embedded (FFPE) sections from patients (median age: 63.8 y, range: 25.9-91.2 y) diagnosed with colon cancer were analyzed. None had received prior chemotherapy.

The samples were all characterized with respect to resistance status, i.e. low (LDR), intermediate (IDR) or extreme (EDR) drug resistance towards a panel of chemotherapeutic drugs, including 5-FU/ Leucovorin, Irinotecan, and Oxaliplatin.

In addition, immunohistochemical data for thymidilate synthase expression, P-glycoprotein expression, p53 mutation status, and DNA ploidity, were available for a number of the tumors.

RNA isolation

Total RNA was isolated by using the miRNEasy FFPE kit from Qiagen.

Microarray profiling (Figure 4)

0.5 µg total RNA was analyzed for miRNA expression on miRCURY LNA™ microRNA Arrays containing T_m normalized capture probes for 2090 miRNAs (Figure 5), including 110 human miRPlus sequences not yet annotated in miRBase, +200 miRNAs discovered by 454 high throughput sequencing, as well as the corresponding pre-miRNAs.
All hybridizations were made against a common reference pool.

Figure 4

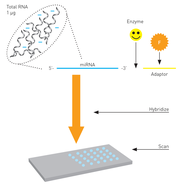


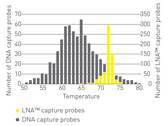
Figure 4. Procedure for miRNA profiling with miRCURY LNA™-based microarrays.
1. Prepare total-RNA
2. Label RNA with Hy5 / Hy3 dyes
3. Hybridize overnight
4. Scan and analyze

Data analysis

MicroRNAs with high discriminatory power between treatments were identified by Welsh T-test (p<0.01). Both unsupervised hierarchical clustering and supervised LDA (linear discriminant analysis) was performed for classifying the samples according to EDR status.

Results – Technology

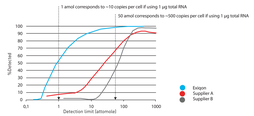
Figure 5



Using LNA™, the T_m is increased significantly and the T_m range is narrowed significantly, com-pared to DNA probes. This results in increased stringency and optimal hybridization conditions for the LNA™ capture probes.

Figure 5. The miRCURY LNA™ enhanced microarray is T_m normalized ensuring that all miRNA targets hybridize to the array with equal affinity under high stringency hybridization conditions.

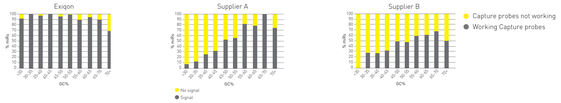
Figure 6



Experimental set-up:
A dilution series of 557 synthetic microRNAs was hybridized, and for each array the percentage of capture probes detecting microRNAs was plotted. At 50 amol only 40% of the probe of Supplier B and 66% of Supplier A detect their targets. Exiqon capture probes detect 96% of their targets. All arrays were processed according to the manufacturers' protocols.

Figure 6. The miRCURY LNA™ enhanced microarray is extremely sensitive

Figure 7



It is clear that Supplier A + B have difficulties in designing optimal probes spanning the entire range of GC%.
In contrast, the miRCURY LNA™ Array offers optimal performance of all probes using T_m-normalized probes.
The unmatched sensitivity and specificity is partly explained by the optimal design of the LNA™ capture probes.
Method: 557 synthetic microRNAs have been used. Each at a concentration of 50 amol (~500 copies per cell if using 1 µg total RNA).

Table 1

GC%	<30	30-35	35-40	40-45	45-50	50-55	55-60	60-65	65-70	70<
# of miRs	15	40	48	91	92	89	39	28	16	12

Results – miRNA profiles

Figure 8

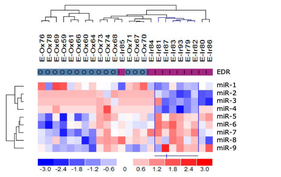


Figure 8. Unsupervised hierarchical clustering based on the 9 miRs (the numbers are arbitrary) that varied most between EDR-Oxaliplatin and EDR-Irinotecan samples (T-test, p<0.01). Clear separation between EDR-Oxaliplatin (left) and EDR-Irinotecan (right) samples is seen; 2 misclassifications (Ir 84-85).

Figure 9

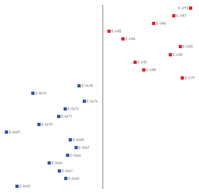


Figure 9. LDA plot showing perfect separationof EDR-irinotecan (red) and EDR-Oxaliplatin (blue) samples.

Figure 10

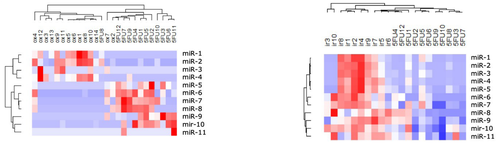


Figure 10. Unsupervised hierarchical clustering based on the 11 miRs (the numbers are arbitrary) that varied most between EDR-5FU and EDR-Oxaliplatin (Ox) and EDR-Irinotecan (Ir) samples (T-test, p<0.01).

Conclusion

- It is possible to determine the EDR status of colorectal cancer based on the tumor's miRNA profile.
- We are currently validating the classifier in an independent test set

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